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Virus-Like 30S RNA in Mouse Cells

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Uninfected JLS-V9 mouse cells are known to express high levels of viral sequences that hybridize to complementary DNA made by the BrdU-induced virus of JLS-V9 cells. The genome in the BrdU-induced virus has been found to consist mainly of an RNA species that migrates as 30S RNA material during electrophoresis through agarose gels. This virus-like 30S RNA, designated VL30 RNA, apparently represents a new class of endogenous defective retroviruses that are not generally evident because of their defectiveness and lack of biological function. Fingerprint analysis and hybridization studies show that VL30 RNA does not have homology with the standard nondefective murine leukemia viruses. Upon superinfection with a nondefective murine leukemia virus, or upon induction of endogenous virus with BrdU, VL30 RNA is rescued into virions by phenotypic mixing. When VL30 RNA is rescued by BrdU induction, the VL30 RNA is mainly organized as a 50S complex, but when VL30 is rescued by superinfection, VL30 is also found in 70S RNA. Rescued VL30 RNA sequences can be reverse transcribed by the virion-associated DNA polymerase in an endogenous reaction. Many mouse cells express the sequences, whereas heterologous cells such as rat or rabbit cells do not contain them. By using hybridization of a complementary DNA probe to cellular RNA immobilized on paper, no subgenomic RNA related to the VL30 RNA could be found in cells expressing the VL30 sequences. From 20 to 50 copies of these sequences were found to be contained in the mouse genome. VL30 RNA is probably present in most stocks of leukemia and sarcoma viruses made in mouse cells.

We have previously shown that the BALB/c-derived cell line JLS-V9 is readily inducible to produce endogenous xenotropic murine leukemia virus ($MuLV_X$) and an ecotropic, N-tropic, non-XC plaque-forming MuLV ($N\text{-}MuLV_E$) (7). Furthermore, it was recognized that the uninduced JLS-V9 cells express high levels of virus-specific sequences in the absence of virus production (7, 14, 17). Here we report biochemical characterization of the particles released from JLS-V9 cells after induction with halogenated pyrimidines. The induced virus is shown to contain a 29-30S RNA that is not related to known MuLV's by sequence homology. This type of virus-like RNA (VL30 RNA) is found to be widely distributed in mouse cells but is not found in heterologous cells; it apparently represents the RNA of a new class of endogenous defective murine retroviruses. Both Howk et al. (22) and Sherwin et al. (31) have reported similar RNA's in other mouse cells.

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MATERIALS AND METHODS

Cells and viruses. NIH/3T3 cells (25) were grown in Dulbecco-modified Eagle medium plus 10% calf serum. SC-1 cells, derived from a wild mouse (16), BALB/c-derived JLS-V9 cells (35), NRK (rat) cells (11), the rabbit cell line SIRC (4), and the mink lung cell line CCL 64 (19) were all grown in Dulbecco-modified Eagle medium plus 10% heat-inactivated fetal calf serum. The following virus-producing cell lines used in this study were derived in this laboratory: NRK cells producing M-MuLV (E. Rothenberg, unpublished data); NIH cells producing M-MuLV (17); NIH cells producing the endogenous N-tropic MuLV ($N\text{-}MuLV_E$) isolated from virus of BrdU-induced JLS-V9 cells (15); mink cells producing an endogenous xenotropic MuLV ($MuLV_X$) isolated by end point dilution of BrdU-induced virus from JLS-V9 cells (5). JLS-V9 cells producing M-MuLV (JLS-V11 cells) (35) were provided by Electronucleonics Inc., Bethesda, Md.; a virus-producing subclone of this culture was used in this study (6).

Preparation of ^{32}P-labeled viral RNA. Cells were rinsed with phosphate-free medium. They were then labeled with phosphate-free medium supplemented with carrier-free [^{32}P]phosphate (0.5 mCi/ml) for 4 h. The labeling medium was removed and dis-

carded, and two 3-h harvests with regular medium were collected. The virus was purified by differential centrifugation and banded in a 25 to 45% sucrose gradient. Virus was lysed with 3.5 M urea–0.15 NaCl–0.01 M Tris (pH 7.4)–0.001 M EDTA–0.5% sodium dodecyl sulfate (SDS), and its RNA was extracted with phenol/chloroform (4% isoamyl alcohol), 1:1, and then ethanol-precipitated according to the method of Bonner and Holmes (21) as described by Sharp et al. (30).

Agarose gel electrophoresis. Isolated viral RNA was suspended in 20 μl of 2 mM sodium acetate–2 mM EDTA–4 mM Tris (pH 7.2), heat denatured in a sealed capillary for 2 min at 80°C, then 5 μl of 50% wt/wt sucrose–0.2% bromophenol blue was added, and electrophoresis was carried out in 1% agarose gels in 5 mM sodium acetate–1 mM EDTA–40 mM Tris-acetate (pH 7.3) at 10 V/cm for 2.5 h as described by Hewlett et al. (20). The gels were stained with ethidium bromide (5 μg/ml) in electrophoresis buffer and observed under UV light. Electrophoresis of RNA on denaturing methylmercuric hydroxide agarose gels was performed as described by Bailey and Davidson (3) with a slab gel apparatus (20 by 12 by 0.3 cm). Methylmercuric hydroxide was obtained from Alfa Products (Danvers, Mass.) as a 1 M stock solution.

Preparation of cytoplasmic RNA's. Cytoplasmic RNA's were prepared as described previously (5). Briefly, cell cultures were trypsinized and washed three times with ice-cold phosphate-buffered saline. Cells were lysed with 5 mM NaCl–0.5 mM $MgCl_2$–0.5% Nonidet P-40–5 mM Tris (pH 7.4) and homogenized with a Dounce homogenizer. Nuclei were removed by centrifugation for 2 min at 2,000 rpm; the supernatant was mixed with an equal volume of 7 M urea–0.3 M NaCl–0.02 M Tris (pH 7.4)–5 mM EDTA–1% SDS; and RNA was extracted with phenol/chloroform (4% isoamyl alcohol), 1:1, ethanol-precipitated, and used for hybridization. Poly(A)-containing RNA was prepared by affinity chromatography on oligo(dT)-cellulose (T3, Collaborative Research).

Preparation of cDNA's and hybridization techniques. ^3H- and ^{32}P-labeled complementary DNAs (cDNA's) were prepared by incubation of the virions in the presence of calf thymus oligodeoxynucleotide primers by the method of Taylor et al. (34). The specific activity of the [^3H]cDNA's was 1 to 2 × 10^7 cpm/μg as calculated from the specific activity of the labeled precursor. Hybridization conditions for the determination of the concentration of virus-specific RNA sequences were as follows. RNA samples were incubated with 500 to 1,000 cpm of [^3H]cDNA in 0.6 M NaCl–0.02 M TES [*N*-tris(hydroxymethyl)methyl-2-aminomethane sulfonic acid, pH 7.4]–2 mM EDTA–0.1% SDS. Reaction mixtures (5 μl each) were sealed in capillaries, boiled for 2 min at 100°C, and then incubated for different lengths of time at 67°C. Unhybridized cDNA was then digested with S1 nuclease for 45 min at 45°C as previously described (14).

Hybridization across agarose gels was carried out as follows. Cylindrical gels of 6 mm diameter were cut into 1-mm slices and put into plastic 1-ml vials (Eppendorf): a 50-μl amount of 500 to 1,000 cpm of [^3H]cDNA in 1.2 M NaCl–0.04 TES (pH 7.4)–4 mM EDTA–0.2% SDS was added, and 50 to 100 μl of Nujol mineral oil was used to cover the sample. The sealed vials were boiled for 5 min at 100°C and then incubated at 68°C. Unhybridized cDNA was digested in 0.5 ml of S1-nuclease reagent (40 U of enzyme per ml, 0.25 M potassium-acetate, pH 4.5, 20 μg of denatured DNA per ml, 0.01 M $ZnSO_4$). Incubation was carried out for 1 h at 45°C. Carrier RNA (0.5 μg) was then added, and the samples were precipitated with trichloroacetic acid and filtered by using two glass fiber filters. The filters were first washed with 5% trichloroacetic acid, then with ethanol-chloroform (1:1) and counted in a scintillation counter. It was imperative that all operations with the molten agarose hybridization mixtures be carried out at greater than 45°C.

Two-dimensional fingerprints of ribonuclease (RNase) T1-resistant oligonucleotides. The fingerprint analysis was carried out as described by Pettersson et al. (25). Briefly, RNA samples were adjusted to a total of 20 μg with yeast RNA. The RNA was collected by ethanol precipitation, washed twice with ethanol, and dried in vacuo. The sample was taken up in a capillary with 8 μl of RNase T1 solution in 20 mM Tris (pH 7.5) and 2 mM EDTA and digested for 1 h at 37°C. After digestion, 4 mg of ultrapure urea (Schwarz Mann) and 5 μl of a dye mixture containing xylene cyanol FF and bromophenol blue (each at 2 mg/ml), 50% (wt/wt) sucrose and 6 M urea were added. The digested RNA was then analyzed by two-dimensional gel electrophoresis as described previously (9, 10). The first dimension was run in 10% acrylamide at pH 3.5 in the presence of 6 M urea, and the second dimension in 21.8% acrylamide at pH 8.

Transfer of RNA from agarose gels to diazobenzyloxymethyl paper. The preparation of diazobenzyloxymethyl paper, the transfer of the RNA from methylmercuric hydroxide agarose gels to this paper, and the hybridization of [^{32}P]cDNA probes with the RNA bound to the paper were carried out as previously described (6) by the method of Alwine et al. (2).

RESULTS

By biological assay, the virions released after BrdU treatment of JLS-V9 cells contain both xenotropic and N-tropic MuLV's (7). After passing these induced viruses to susceptible cells, their genomes were found to be the usual 70S RNA of retroviruses (data not shown). When BrdU-treated JLS-V9 cells were directly labeled with $^{32}PO_4$, however, the majority of the RNA in released particles (BU-V9 virus) did not sediment at 70S, but rather sedimented as a broad band centered at about 50S (Fig. 1).

After heat denaturation, the subunit sizes of the genome RNA's of the $MuLV_X$ and the N-$MuLV_E$ derived from the BU-V9 virus were 38S (data not shown). To investigate the subunit size of the RNA directly isolated from BU-V9 virus, the RNA was heat denatured and subjected to electrophoresis through a 1% agarose gel (Fig. 2). The majority of the RNA migrated as 29 to 30S RNA, and very little had the size of the 38S RNA from the biologically active viruses.

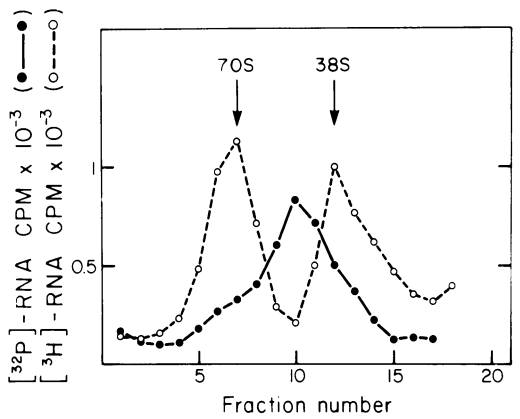

FIG. 1. *RNA from BrdU-induced JLS-V9 cells. JLS-V9 cells were seeded at 2 × 10⁶ cells per 15-cm plate. On the next day, the medium was replaced with medium containing BrdU and deoxycytidine, each at 20 μg/ml. After 24 h, the BrdU-containing medium was replaced with regular medium. After a further 26-h period, the cultures were labeled with $^{32}PO_4$. The labeled virus was purified, and the RNA was extracted as described in the text. The RNA was suspended in 2 mM EDTA–5 mM Tris (pH 7.4)–0.5% SDS and was layered onto an 11-ml, 15 to 30% sucrose gradient in 0.1 M NaCl–0.001 M EDTA–0.05 M Tris (pH 7.3)–1% SDS. Centrifugation was for 3.5 h in an SW41 rotor at 35,000 rpm, and 0.5-ml fractions were collected. 70S and 38S [^{3}H]M-MuLV RNA were included as size markers.*

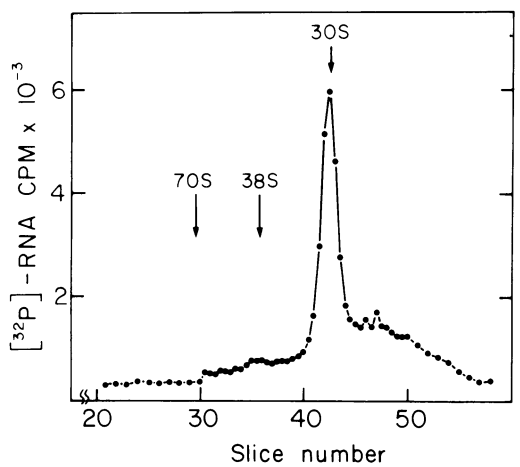

FIG. 2. *Agarose gel electrophoresis of denatured RNA from virions produced by BrdU-induced JLS-V9 cells. ^{32}P-labeled BU-V9 RNA was heat denatured and subjected to electrophoresis in a 1% agarose tube gel for 2.5 h at 10 V/cm; 0.1 μg of 70S and 38S M-MuLV were included as size markers. The gel was stained with ethidium bromide, the position of the markers was determined and the gel was then cut into 1-mm slices. The amount of ^{32}P was determined in the individual slices.*

The particles induced from JLS-V9 cells thus contain mainly 29 to 30S RNA (referred to here as "virus-like 30S RNA" [VL30 RNA]) and not, as one might have expected, 38S genomic RNA corresponding to the BALB/c endogenous $MuLV_X$ and N-tropic MuLV. We call the RNA virus-like because it can be packaged in virus particles, can polymerize to a 50S form and, as seen below, can be reverse transcribed.

Mouse cells express sequences related to the VL30 RNA. We have previously made radioactive cDNA from the BU-V9 virus by using the virion-associated reverse transcriptase activity (6). To investigate what size RNA in the preparation was template for this cDNA, individual slices of an agarose gel were hybridized with BU-V9 cDNA (Fig. 3). The cDNA hybridized mainly to 30S RNA with little hybridization to the 38S RNA. This cDNA can therefore be used to study the expression of VL30 RNA in various cells.

As shown previously, uninduced JLS-V9 cells express high levels of RNA complementary to BU-V9 cDNA (6, 15). Annealing experiments of BU-V9 cDNA to cytoplasmic RNA's from other mouse cells revealed that VL30 RNA sequences are not only expressed in JLS-V9 cells but also

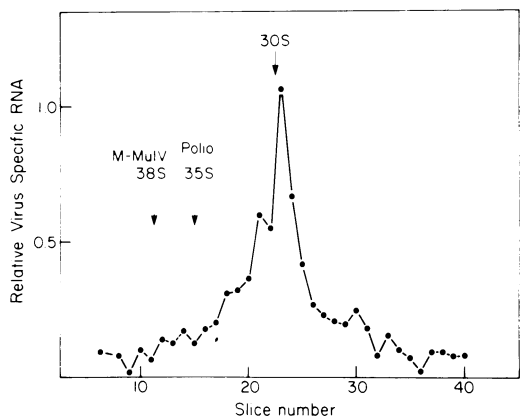

FIG. 3. *Hybridization of BU-V9 viral RNA with BU-V9 cDNA. About 0.1 μg of BU-V9 viral RNA was heat denatured and subjected to electrophoresis in a 1% agarose tube gel as described in the legend to Fig. 2. ^{32}P-labeled poliovirus RNA was included as internal size marker. The location of the BU-V9 viral RNA was determined by ethidium bromide staining. The gel was cut into 1-mm slices. The position of the ^{32}P-poliovirus marker was determined by counting Cerenkov radiation. The position of denatured M-MuLV RNA relative to the poliovirus RNA was determined in a separate parallel tube gel. The gel slices were hybridized with BU-V9 cDNA as described in the text, and the relative virus-specific RNA concentration was determined as described by Fan and Baltimore (14).*

in other mouse cells (Table 1). They are not, however, detectable in heterologous cells such as rat (NRK), rabbit (SIRC), or mink cells. The lack of homology to NRK RNA indicates that the VL30 RNA sequences are not related to those in a physically similar 30S RNA in rat cells (27-29).

VL30 RNA sequences are rescued by phenotypic mixing. Because uninduced JLS-V9 cells produce no particles, it is likely that the helper-independent viruses induced by BrdU provide at least one critical function necessary to put VL30 RNA into particles. We might then expect the VL30 sequences to be rescued by superinfection of JLS-V9 cells with Moloney MuLV (M-MuLV). The RNA from particles produced by M-MuLV-infected JLS-V9 cells were therefore analyzed by sedimentation in an SDS sucrose gradient and, after heat denaturation, by agarose gel electrophoresis (Fig. 4). The native RNA had a major 70S peak and a shoulder at about 50S (Fig. 4A). When this was pooled and heat denatured, a major 30S RNA was found with about 30% migrating at 38S (Fig. 4B).

To compare the VL30 RNA contained in BU-V9 virus with the RNA's in particles produced by M-MuLV-infected JLS-V9 cells, RNA fingerprints of RNase T1-resistant oligonucleotides were compared. The 50S RNA of the BU-V9 virus and the 50S RNA from particles made by M-MuLV-infected JLS-V9 cells were digested with RNase T1 and then analyzed by two-dimensional gel electrophoresis as described by Fiers and De Wachter (10) and Coffin and Billeter (9) (Fig. 5). The BU-V9 virus gave rise to a very distinct pattern of large RNase T1-resistant oligonucleotides (Fig. 5A), different from those of M-MuLV 38S RNA (Fig. 5C). The intensity of some of the spots of apparently equal length in the BU-V9 fingerprint varied, indicating possible heterogeneity. All of the characteristic large oligonucleotides of the fingerprint of the 50S RNA from the M-MuLV produced by the JLS-V9 cells were contained in the fingerprint of the BU-V9 virus (Fig. 5B).

TABLE 1. *Expression of VL30 RNA in mouse and heterologous cells*[a]

Cell line	Extent hybridized[b]	$C_rt_{1/2}$ (mol s/liter)[c]
JLS-V9	71	40
JLS-V9, BrdU treated	100	6
NIH/3T3	51	200
SC-1 (wild mouse)	65	ND
SIRC (rabbit)	6	ND
NRK (rat)	5	ND

[a] BU-V9 [^{3}H]cDNA was annealed with cytoplasmic RNA of the indicated cell lines. Annealings were performed to a C_rt (product of initial RNA concentration and time of incubation) of at least 5×10^3 mol s/liter.

[b] Values represent percentage of trichloroacetic acid-precipitable radioactivity which is resistant to S1 nuclease.

[c] ND, Not determined.

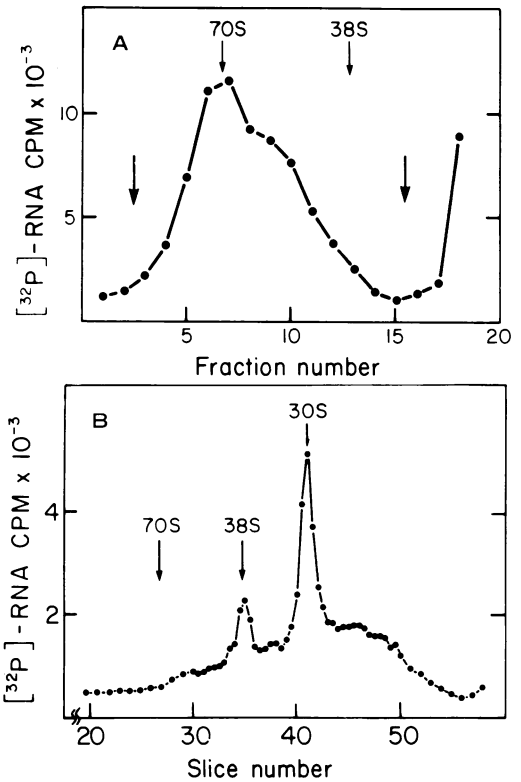

FIG. 4. *Rescue of VL30 by superinfection. Virus from M-MuLV-infected JLS-V9 cells was labeled with [^{32}P]phosphoric acid and the RNA extracted as described in the text. (A) The RNA was then sedimented in a 15 to 30% SDS-sucrose gradient for 3.5 h in an SW41 rotor as described in the legend to Fig. 1. The positions of 70S and 38S were determined in a parallel gradient containing the ^{32}P-viral RNA and ^{3}H-labeled 70S and 38S M-MuLV RNA. (B) The regions of the sucrose gradient between the unlabeled arrows were pooled, heat denatured, and subjected to electrophoresis through a 1% agarose tube gel. About 0.1 μg of 70S and 38S M-MuLV were included as internal size markers.*

RNA's from N-tropic MuLV and M-MuLV produced by NIH cells were also analyzed by gel electrophoresis (Fig. 6). As expected, both viruses contained 30S RNA: 50% in the N-tropic MuLV stock and only a small amount in the M-MuLV stock. Hybridization of individual gel slices of an agarose gel of N-tropic MuLV RNA with cDNA probes made from either BU-V9 virus or AKR virus showed that the 38S RNA hybridized to the AKR cDNA and the 30S com-

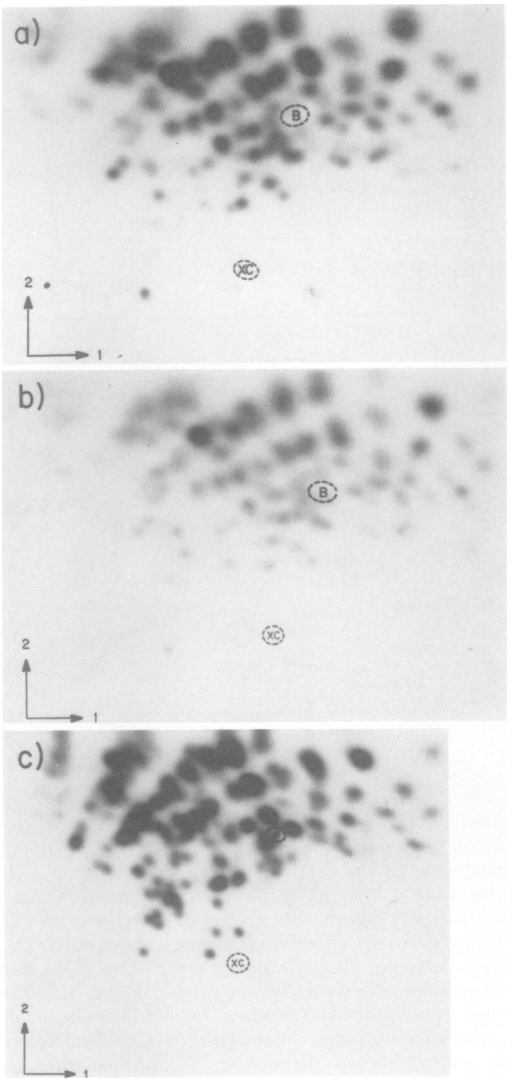

FIG. 5. *Two-dimensional gel electrophoresis of ^{32}P-labeled T1 oligonucleotides from BU-V9 virus and the VL30 RNA from M-MuLV produced in JLS-V9 cells, (a) RNase T1 digest of "50S" complex RNA of ^{32}P-labeled BU-V9 RNA (see Fig. 1). (b) RNase T1 digest of 55S RNA of ^{32}P-labeled M-MuLV produced in JLS-V9 cells (see Fig. 4). (c) RNase T1 digest of 70S RNA of ^{32}P-labeled M-MuLV produced in NIH cells. Symbols: B, bromophenol blue dye; XC, xylene cyanol dye. Origin for the second dimension is indicated by the arrow showing the direction of migration of the first dimension.*

ponent to BU-V9 cDNA (Fig. 7). These experiments provide evidence that VL30 RNA sequences are often rescued by phenotypic mixing with helper MuLV's when a producer mouse cell line is made by infection with an exogenous virus. The amount of VL30 in different virus preparations appears to reflect the amount of VL30 expressed in the respective cell lines.

Undenatured RNA from the N-tropic MuLV produced by NIH cells sedimented as 70S and no 50S material was observed. Upon denaturation equal amounts of 38S and 30S RNA were generated. As mentioned above, the undena-

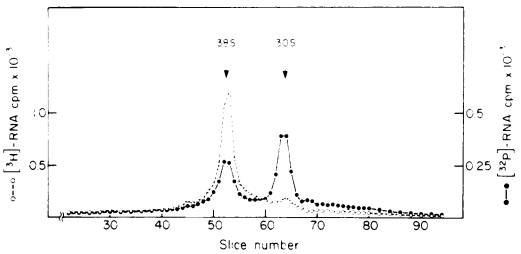

FIG. 6. *VL30 RNA in virus produced by NIH cells. ^{32}P-labeled RNA from M-MuLV (○) and N-tropic MuLV (●) produced in NIH cells was heat denatured and subjected to electrophoresis through 1% agarose gels.*

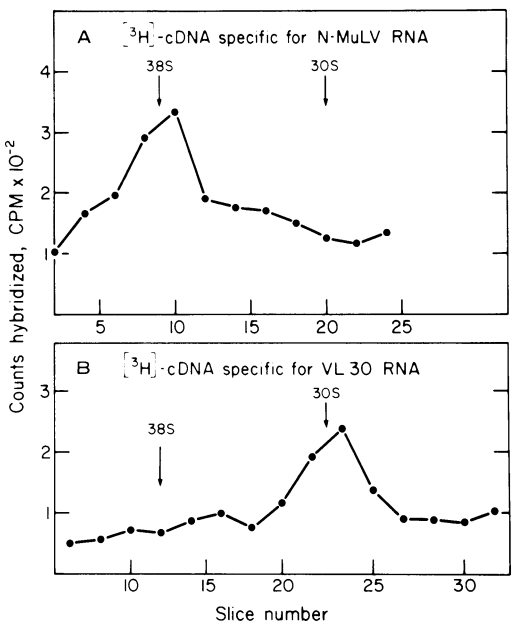

FIG. 7. *Hybridization specificity of the 38S and 30S RNAs in an N-tropic MuLV stock produced by NIH/3T3 cells. About 0.1 μg of N-tropic MuLV RNA from cells was heat denatured and subjected to electrophoresis through a 1% agarose gel. The positions of the 38S and 30S subunits were localized by ethidium bromide staining. The gel was cut into 1-mm slices. Alternate slices were hybridized with (A) [^{3}H]cDNA made from N-tropic MuLV for 6 h and (B) [^{3}H]cDNA from BU-V9 virus for 44 h. The extent of hybridization was determined as described in the text.*

tured RNA from M-MuLV produced by JLS-V9 cells consisted of two-thirds that sedimented as 70S and one-third that sedimented as 50S; upon denaturation, it consisted of one-third 38S and two-thirds 30S RNA. Undenatured BU-V9 viral RNA consists mostly of 50S material that is denatured to 30S RNA.

Maisel and co-workers (24) have recently described a similar phenomenon with the MuLV-murine sarcoma virus complex. They also found both 38S and 30S molecules sedimenting with 70S material. Electron microscopic analysis revealed only homodimers and no heterodimers. No dimers of 30S RNA were found to be sedimenting as 70S material if the cells were not producing any substantial amount of helper virus. It is interesting to note that both the VL30 RNA described here and the murine sarcoma virus genome RNA form these fast-sedimenting dimer structures dependent on the presence of helper virus.

The expression of VL30 RNA sequences is enhanced by inducing agents. In a previous study of the induction of viral sequences in JLS-V9 cells, we found that expression of viral sequences was enhanced five- to tenfold upon induction with halogenated pyrimidines (7). In retrospect, the cDNA used in that study was cDNA to the VL30 RNA. This experiment therefore showed that induction of JLS-V9 cells with halogenated pyrimidines enhances the expression of VL30 sequences.

Sequence homology of VL30 sequences with eco- and xenotropic MuLV's. From the fingerprints in Fig. 5, the lack of relationship between VL30 and M-MuLV 38S RNA is evident. To investigate the relationship of VL30 to other MuLV's, hybridization probes were made to M-MuLV and to the endogenous viruses of BALB/c mice—N-tropic BALB:virus-1 ($MuLV_E$) and xenotropic BALB:virus-2 ($MuLV_X$) (1). To represent BALB:virus-1, for convenience a cDNA to ecotropic AKR MuLV was used; such a cDNA hybridizes equally to $MuLV_E$-BALB and $MuLV_E$-AKR RNA (Table 2). The M-MuLV and xenotropic viruses were grown in heterologous cells to avoid contamination of these MuLV probes with VL30 sequences. M-MuLV cDNA was made from M-MuLV grown in NRK cells (clone CP1), and $MuLV_X$ cDNA was made from virus grown in mink cells. The cDNA's were prepared by incubation of the virions in the presence of exogenously added calf thymus oligodeoxynucleotide primers, a condition that produces a quite uniformly representative cDNA (34). Therefore, the plateau levels of hybridization between a given cDNA and cytoplasmic RNA preparations could be used to estimate the approximate cross homology between the virus specifying the cDNA and the cellular RNA's. Previously we had shown that uninduced JLS-V9 RNA could not protect cDNA from the N-tropic MuLV BALB/c mice (17). In the present study, uninduced JLS-V9 cell RNA protected only 8% of the xenotropic MuLV cDNA, 5% of M-MuLV cDNA, and 10% of the ecotropic AKR MuLV cDNA (Table 2). In agreement with the results of others, the cross-homologies between the M-MuLV ecotropic and xenotropic endogenous viruses of BALB/c mice were 50 to 75% (8). These results indicate that VL30 is at most weakly related to the nondefective MuLV's tested and suggests that VL30 represents sequences from an agent totally unrelated to the standard murine type C viruses.

The cDNA made by BU-V9 virus was protected to 70% by uninduced JLS-V9 RNA, to 25% by RNA from mink cells producing $MuLV_X$ and to 100% by RNA from BrdU-induced JLS-V9 cells (Table 2). Apparently, about 25% of the cDNA made from BU-V9 virus is complementary to $MuLV_X$, and the remainder is complementary to VL30 RNA. As indicated by the data of Table 1, $MuLV_X$ RNA is not evident in JLS-V9 cells before induction with BrdU. The lower extent of hybridization of BU-V9 cDNA by NIH and wild mouse cell RNA as compared to JLS-V9 RNA may imply heterogeneity in VL30.

VL30 sequences are contained in multiple copies in the mouse genome. To determine how many copies of the VL30 sequences

TABLE 2. *Hybridization of VL30 sequences in JLS-V9 cells to cDNA from endogenous murine viruses*

[^{3}H]cDNA source	Cytoplasmic RNA from[a]				
	NIH ($MuLV_E$-BALB)	NIH ($MuLV_E$-AKR)	NIH (M-MuLV)	Mink ($MuLV_X$)	JLS-V9
$MuLV_E$-AKR	90	90	60	50	10
$MuLV_X$	ND	55	55	75	8
M-MuLV	ND	ND	67	ND	5
BU-V9	ND	ND	ND	25	70

[a] The viruses used to infect the cells from which the RNA was extracted are shown in parentheses; NIH indicates NIH/3T3 cells. Values represent percent labeled cDNA hybridizing to a saturating amount of cytoplasmic RNA from the indicated cells. ND, Not determined.

are contained in the mouse genome, a C_0t analysis was carried out with BALB/c mouse liver DNA and [^{3}H]VL30 cDNA. This analysis showed that the VL30 sequences reanneal at a $C_0t_{1/2}$ (product of initial DNA concentration and time of hybridization for one-half completion of the reaction) of about 50 (data not shown); thus, 20 to 50 copies of these sequences are contained in the mouse genome in agreement with the data of Howk et al. (22).

Size of VL30-specific cytoplasmic RNA. Cells producing retroviruses have been recognized to contain genome-sized RNA as well as smaller RNA's that can serve as mRNA for the production of virus-coded proteins. In a previous study, we have analyzed the size of what is now recognized as VL30-specific sequences in JLS-V9 cells (17). With the availability of the new and powerful RNA "blotting" technique developed by Alwine et al. (2), we have reinvestigated this question. Poly(A)-selected cytoplasmic RNA from JLS-V9 cells, JLS-V11 cells and NIH/3T3 cells were subjected to electrophoresis through a denaturing methylmercuric hydroxide agarose gel. The RNA was transferred from the agarose gel by the method of Southern (33) to diazobenzyloxymethyl paper, an affinity paper for nucleic acids (2). The virus-specific RNA that was covalently linked to the paper was then detected by hybridization with [^{32}P]VL30 cDNA and with [^{32}P]M-MuLV cDNA made with M-MuLV from NRK cells (Fig. 8). When hybridized with [^{32}P]VL30 cDNA, the only band visible in JLS-V9 RNA (lane c) after a short exposure was one comigrating with the VL30 virion RNA (lanes a and e); upon 10-fold longer exposure (lane g), no other RNA species was detected in this RNA sample. With M-MuLV-producing JLS-V11 cells, after a short exposure (lane d) only 30S material was detectable, but after a longer exposure (lane h) 38S, 36S, and 21S M-MuLV RNA species were also detected. These RNA's have been separately characterized as, respectively, genome-size RNA, a deleted M-MuLV RNA, and the mRNA for viral glycoprotein synthesis (6).

Uninfected NIH cell cytoplasmic RNA (lanes b and f) also contains 30S material detectable with the VL30 cDNA. No subgenomic RNA species related to VL30 sequences was evident in cytoplasmic RNA from JLS-V9, NIH, or JLS-V11 cells within the detection limits of this method. After hybridization with [^{32}P]M-MuLV cDNA made from virus produced in rat cells, no JLS-V9 RNA reacted after short (lane j) or after long exposure (data not shown). With JLS-V11 RNA only the M-MuLV 38S, 36S, and 21S RNA's were detected. Hybridization with the M-MuLV probe showed again the lack of sequence homology between M-MuLV and VL30 RNA. Hybridization of the VL30 cDNA to M-MuLV 38S, 36S, and 21S RNA in lane h therefore indicates that our VL30 cDNA is not pure and contains endogenous helper MuLV sequences.

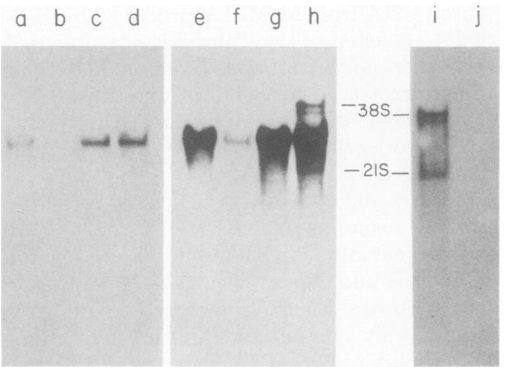

FIG. 8. *Electrophoretic analyses of VL30 sequences in the cytoplasm of cells. Poly(A)-selected cytoplasmic RNA from JLS-V9 cells, NIH cells, and JLS-V11 cells (5 μg each) were electrophoretically separated on a 1% agarose methylmercuric hydroxide gel for 2.5 h at 6 V/cm. The RNA in the gel was then transferred to diazobenzyloxymethyl paper, and VL30-specific RNA sequences were visualized by hybridization for 30 h with [^{32}P]cDNA made from BrdU-induced virus of JLS-V9 cells (lanes a–h). For lanes i and j, [^{32}P]cDNA made from M-MuLV grown in NRK (rat) cells was used. The exposure time for the autoradiography was 8 h (lanes a–d and i, j) and 70 h (lanes e–h). Lanes c, g, and j contained JLS-V9 RNA, lanes d, h, and i contained JLS-V11 RNA; lanes b and f contained NIH RNA, and lanes a and e contained RNA from the induced virus from JLS-V9 cells.*

DISCUSSION

Our studies and the work recently published by Howk et al. (22), Sherwin et al. (31), and Duesberg and Scolnick (12) demonstrate a new endogenous virus-like RNA in mouse cells that is unrelated to that in standard C-type viruses. They also provide a cautionary note that MuLV stocks made on mouse cells will usually contain a previously unrecognized component (VL30 RNA). That VL30 RNA is not related to the RNA of standard MuLV's is shown by the lack of T1 oligonucleotides in common between M-MuLV and VL30 RNA and by the lack of hybridization of RNA in JLS-V9 cells with cDNA made from virions from M-MuLV, the N-tropic MuLV and the xenotropic MuLV from BALB/c mice. The cDNA used in the study of these sequences by Howk et al. (22) was derived from MuLV grown on NIH mouse cells and the one used by Sherwin et al. (31) from virus grown on

SC-1 wild mouse cells. Our VL30 cDNA hybridizes also with RNA from NIH and from SC-1 cells, and we therefore believe that the sequences described here are closely related to those reported by others. In fact, Sherwin et al. (31) used cDNA made in our laboratory from BU-V9 virus to demonstrate the close relationship of the sequences found in the different mouse strains. We assume that most mouse strains carry the genes for making VL30 and that many mouse cell lines express VL30 sequences.

In our previous studies characterizing endogenous viral sequences in JLS-V9 cells, we reported that these cells express high levels of viral sequences. These sequences were detected with cDNA probes made by M-MuLV produced either by JLS-V11 cells or by BU-V9 virus (7, 15, 17). In interpreting the earlier work, we were not aware that VL30 RNA existed and assumed that the sequences were derived from endogenous C-type viruses related to the standard MuLV's. We showed that the sequences did not derive from N-tropic MuLV (15) but seemed to derive from $MuLV_X$, because the main biologically active entity in BU-V9 virion preparations is $MuLV_X$ (7); we now know that they are unrelated to $MuLV_X$ also (Table 2) and therefore they represent a new class of murine retroviruses. Furthermore, we can say that JLS-V9 cells express very little N-tropic MuLV or $MuLV_X$-related sequences as RNA but that both are induced after BrdU treatment (7, 16). We had previously reported that JLS-V9 cells make RNA's sedimenting at both 38S and 27S; we interpreted these as probably containing different sequences. The 38S RNA, however, is not evident after total denaturation (Fig. 8), and we now believe it to be an aggregation artifact. The RNA previously called 27S is presumably VL30 RNA. From that previous work we know it to contain poly(A) (15). Because no smaller discrete RNA is present in JLS-V9 cells, the presence of VL30 RNA in polyribosomes (16) suggests that it is the only mRNA for translation of the proteins encoded by the VL30 provirus. Such proteins are being sought.

We call the VL30 sequences "virus-like" because they can aggregate to a faster sedimenting form (50S) like the 70S RNA of the standard MuLV's. Similarly, as described for the murine sarcoma virus 30S RNA by Maisel et al. (24), VL30 sequences are also found to sediment as 70S material if the virions contain a significant amount of standard MuLV RNA. The VL30 sequences can be reverse transcribed in an endogenous reaction and they are readily packaged by type-C viruses. VL30 RNA may or may not represent defective viral genomes; this is uncertain because we have no evidence of a nondefective virus related to VL30 RNA. If it is a defective virus, it could be defective partly because no subgenomic RNA's can apparently be generated from the 30S RNA. Possibly the sequences essential for the splicing event that generates 21S RNA (13, 26) have been lost. We previously found that what now is recognized as VL30 RNA is present on polyribosomes in JLS-V9 cells (16), but these cells are negative for all murine C-type viral proteins assayed (6). Thus, we do not know whether these sequences contain information for the synthesis of viral proteins. Whatever the VL30 sequences represent, the physical behavior of the VL30 RNA is so much like that of the genome of C-type viruses that it seems appropriate to call them virus-like.

No C-type virus stock made on a mouse cell line can be assured of lacking VL30 RNA. This must be taken into account when carrying out hybridization experiments with MuLV's. Because rat cells contain an unrelated 30S RNA with similar properties and other cells could well have their own virus-like RNA's, all hybridization experiments must control for such contaminants.

There is evidence that Harvey and Kirsten sarcoma viruses have been generated by recombination of rat 30S RNA and a standard MuLV (31). It seemed possible that the VL30 sequences could have been involved in the generation of mouse-derived transforming viruses such as Abelson virus, Moloney sarcoma virus, and BALB murine sarcoma virus. We were, however, unable to detect VL30 sequences in these viruses (P. Besmer, unpublished data) in agreement with Howk et al. (22).

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